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## THE St-B17 SEROTONIN RECEPTOR
### Scope of the Invention

This invention relates to cloning and characterization of cellular receptors. Specifically, this invention relates to the cloning and characterization of the St-B17 serotonin receptor protein.

### Background

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) has a variety of functions in the central nervous system. It has been implicated in many cognitive and behavioral functions, including aggression, sexual behavior, learning and sleep. Disruptions of serotonergic systems may be a critical factor in a number of clinical disorders or conditions including schizophrenia, depression, obsessive compulsive disorder, anxiety, migraine headaches, and pain.

The multitude of effects produced by serotonin are mediated by various serotonin receptors which exist in the central and peripheral nervous system. The transduction of serotonergic signals across the neuronal membrane is mediated by a diversity of receptor subtypes which, in mammals, appear to fall into four pharmacologically distinct classes designated $5\text{-HT}_1$ - $5\text{-HT}_4$. The $5HT_1$ subcategory has been further subdivided into five different subtypes referred to as $5HT_{1A-E}$. The primary structures for a number of these receptors have been elucidated by molecular cloning, including the $5\text{-HT}_1$, $5\text{-HT}_2$ and $5HT_3$ subclasses. In addition, the sequences of three different Drosophila serotonin receptors, $5\text{-HT}_{dro1}$ and $5\text{-HT}_{dro2A,B}$, have been reported.

Selective therapeutic agents, including agonist and antagonist drugs, have been developed based on serotonin receptor technologies. $5\text{-HT}_2$ antagonists, for example, are useful in the treatment of schizophrenia, parkinsonism, and anxiety disorders. Several azapirones, such as buspirone, gepirone, and ipsapirone, have high affinities for $5HT_{1A}$ receptors in the brain, and are useful in the treatment of anxiety. Highly selective 5-HT uptake inhibitors, which have minimal effects on norepinephrine or dopamine uptake or on

other neurotransmitter receptors, have been used to successfully treat depression.

Characterization of all specific 5-HT receptors would clarify the role of serotonin in the central nervous system. Analysis of the receptor subtypes and their functional role in the central nervous system would help elucidate the pathophysiological basis of many human diseases. Accordingly, there is a need for identification and characterization of all specific 5-HT receptors, and the development of selective therapeutic agents based on these receptor technologies.

## Description of the Figures

**Figure 1:** Saturation analysis of specified [$^{125}$I]-LSD binding to membranes prepared from Cos-7 cells transiently transfected with construct pSRα-B17. The experiment shown is representative of 3 independent experiments conducted in triplicate. Inset, scatchard analysis of saturation binding data. In this experiment the $K_D$ and $B_{max}$ values were 1.5 nM and 3.4 pMoles mg protein$^{-1}$, respectively.

**Figure 2:** Pharmacological analysis of [$^{125}$I]-LSD binding to Cos-7 cell membranes expressing clone St-B17. Competition curves shown are representative of 3 independent experiments conducted in triplicate.

**Figure 3:** Northern blot analysis of St-B17 RNA transcripts in rat brain. Each lane contained 3 μg of poly (A+) RNA. Lanes: AMYG, amygdala; CB, cerebellum; CTX, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; MED, medulla; OB, olfactory bulb; OT, olfactory tubercle; PIT, pituitary; RET, retina; STR, striatum; THAL, thalamus. Locations of RNA size (kb) markers are indicated.

**Figure 4:** Accumulation of cAMP in response to agonist activation of St-B17 stably expressed in HEK-293 cells. This figure shows a dose-response curve activation of St-B17 by serotonin in stably transfected, intact HEK-293 cells.

**Figure 5:** Pharmacological analysis of serotonin stimulated cAMP accumulation in stably transfected HEK-293 cells. DE is an acronym for dihydroergocryptine. All drugs were assayed at 10 μM final concentration.

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## Summary

One embodiment of the present invention is the isolated mammalian serotonin receptor protein St-B17. Preferably this receptor protein is human. The present invention also encompasses species variations of the St-B17 receptor.

Another embodiment of the present invention is a method for screening a drug candidate for central nervous system activity by contacting the drug candidate with the St-B17 protein and measuring binding of the drug candidate by the protein.

An additional embodiment of the present invention is a method for screening a drug candidate for central nervous system activity by first contacting the St-B17 serotonin receptor protein with a first molecule known to be bound by the protein to form a first complex of the protein and the first molecule; then contacting the first complex with the drug candidate; and finally measuring whether the drug candidate displaces the first molecule from the first complex. This method can preferably include in the measuring step, the step of measuring the formation of a second complex of the protein and the drug candidate. An alternative method of measuring the displacement of the first molecule can be performed by measuring the amount of the first molecule that is not bound to the protein.

A further embodiment of the present invention includes a recombinant construct of the polynucleotide, preferably the polynucleotide of SEQ ID NO: 7, encoding the St-B17 serotonin receptor protein operably linked to a heterologous promoter.

Another embodiment of the present invention is directed at the polynucleotide sequence encoding the St-B17 receptor. Preferably, this polynucleotide sequence is Sequence ID NO: 7 or species variations thereof. Additionally, the present invention encompasses an isolated nucleotide sequence having homology to at least 18 contiguous nucleotides of the St-B17 gene.

The present invention also embodies a mammalian cell line, preferably human, in continuous culture expressing the

St-B17 serotonin receptor protein. Particularly, the cell line contains the polynucleotide Sequence ID NO: 7 or species variations thereof. One especially preferred embodiment includes HEK 293 cells in continuous culture expressing the St-B17 receptor.

Yet another embodiment of the present invention includes isolated antibodies against the St-B17 serotonin receptor protein. Preferably these isolated antibodies are polyclonal, even more preferably, the isolated antibodies are monoclonal.

An even further embodiment of the present invention includes a method of screening a ligand for binding to the St-B17 serotonin receptor by the steps of:

transfecting a cell line with a gene encoding the St-B17 serotonin receptor protein in an expression vector;

culturing the cell line to express the gene in media containing a ligand of the receptor; and

measuring the binding of the ligand the receptor produced by the cell line.

Preferably, in this method, the cell line is from a mammal, more preferably a human, and most preferably the cell line is HEK 293. An additional preferred embodiment of the above method entails transfecting cells with the St-B17 serotonin receptor encoded by the polynucleotide of SEQ ID NO: 7. Additional preferred embodiments of this method include using eukaryotic expression vectors, preferably pSRα-B17. Another preferred embodiment includes using ligands that are labeled and the label is either radioactive or colorimetric.

Still another embodiment of the present invention is a method of determining the ability of a drug to inhibit ligand binding to the St-B17 serotonin receptor protein by the following steps:

transfecting a cell line with a DNA sequence encoding St-B17 in an expression vector;

culturing the cell line to express the St-B17 receptor in media containing a ligand of the receptor;

determining the level of binding of the ligand
to the receptor;

culturing the same cell line to express the
receptor in the presence of both the ligand and the
drug; and

determining the level of binding of the ligand
to the expressed receptor, wherein a lower level of
binding in the presence of the drug indicates that
the compound is an inhibitor of ligand binding.

Preferably, the cell line of this method is mammalian, most
preferably HEK 293, and the ligand, the drug or both are
labeled. Additionally, the expression vector of this method
can preferably be pSRα-B17 and the ligand can advantageously
be serotonin.

Yet another embodiment of the present invention is a
method for determining the ability of a drug to inhibit ligand
binding to the St-B17 serotonin receptor by the following
steps:

transfecting a cell line with a gene encoding
the St-B17 receptor protein in an expression
vector;

culturing the cell line to express the
receptor;

determining the level of binding of the ligand
to the receptor;

culturing the same cell line to express the
receptor in the presence of both the ligand and the
drug being tested for inhibition; and

determining the level of cAMP accumulation in
the cell, wherein a lower level of cAMP
accumulation in the cell in the presence of the
drug indicates inhibition of ligand binding.

Still another embodiment of the present invention is a
method of determining the ability of a drug to inhibit ligand
binding to the St-B17 serotonin receptor protein by the steps
of:

transfecting a cell line with a DNA sequence encoding St-B17 in an expression vector;

culturing the cell line to express the St-B17 receptor in media containing a ligand of the receptor;

isolating the membranes from the cell line, wherein the membranes contain the expressed St-B17 receptor;

determining the level of binding of the ligand to the receptor on the membranes;

incubating the membranes in the presence of both the ligand and the drug; and

determining the level of binding of the ligand to the membranes, wherein a lower level of binding in the presence of the drug indicates that the compound is an inhibitor of ligand binding.

Still another embodiment of the present invention is a method for detecting expression of the St-B17 serotonin receptor gene in a tissue sample by the steps of:

removing a tissue sample from a mammal;

contacting the mRNA in the cytoplasm of the tissue sample with a labeled polynucleotide probe having homology to the St-B17 serotonin receptor gene; and

detecting the binding of the probe to the tissue wherein positive binding indicates expression of the St-B17 serotonin receptor gene.

## Detailed Description

We have cloned a new serotonin 5-HT (St-B17) receptor gene from rat and human brain cells. This receptor has a nucleotide sequence that is distinct from any previous gene in the serotonin family. The rat St-B17 gene has a 1311 bp open reading frame coding for a protein with 437 amino acids. The human St-B17 gene has a 1320 bp open reading frame coding for a protein with 440 amino acids.

In addition, this receptor protein exhibits a distinct pharmacological profile not previously described. The unique

pharmacology together with the relatively low level of homology (<50%) of St-B17 with previously cloned 5-HT receptor subtypes indicates that this receptor does not belong to any of the previously defined $5\text{-HT}_1$ - $5\text{-HT}_4$ subcategories of 5-HT receptors. Thus, St-B17 represents an unknown and uncharacterized receptor.

The molecular cloning of St-B17 will be useful for developing drugs which interact with 5-HT receptors. For instance, the cloning of this novel gene allowed us to develop new assays for discovering serotonin receptor binding and inhibitory drugs. These assays were performed by transfecting cells, such as Cos-7 mammalian cells, with the gene encoding the St-B17 serotonin receptor protein in an expression plasmid. The Cos-7 cells were then placed in media and expressed the St-B17 receptor on their cell surface. Radiolabeled ligands were then tested for binding to these transfected cells.

By using this method we were able to screen many drugs for their binding activity to the serotonin receptor. Similarly, this assay system was used to detect competitive inhibitors of serotonin binding. By incubating the transfected cells in the presence of a radiolabeled known binding molecule (LSD) we were able to test various drugs for their ability to inhibit this binding. Table 1 below describes a suite of drugs that had wide-ranging affects on $^{125}\text{I}$-LSD binding to the rat St-B17 serotonin receptor.

Since serotonin binding is intimately involved in the functionality of the central nervous system, assays for detecting drugs which bind or block serotonin receptors are of paramount importance. As discussed in the background, many behavioral functions are believed to be mediated through serotonergic systems. For researchers to be able to accurately assess the projected effects of a new serotonin-related drug in humans, it is important to test that drug's effect on every known serotonin receptor. The discovery of the St-B17 receptor provides the researcher with a previously

unknown assay to study possible *in vivo* effects of the drug being tested.

It is also anticipated that isolated St-B17 receptor protein will be useful for performing assays to determine agents which bind or inhibit binding to serotonin receptors. In discussing the isolated St-B17 receptor protein, we are referring to not only affinity-purified protein, but also receptor proteins bound in a membrane and removed from the cell. For example, we transfected Cos-7 cells with the gene encoding the St-B17 receptor protein in an expression plasmid. After incubation in media to allow St-B17 receptor expression the cells were lysed and the membranes (containing the bound St-B17 receptors) were pelleted. These membranes, isolated from the host cells were used for radiolabeled ligand binding studies.

The present invention includes isolated St-B17 serotonin receptors from rats, humans, other mammals, and other vertebrates. Further investigations of St-B17's role *in vivo* and *in vitro* will help improve current therapies for several neuropsychiatric disorders by providing additional information on each drug's receptor-mediated action.

For the first step in cloning the St-B17 receptor we used the polymerase chain reaction (PCR) to selectively amplify cDNA sequences from mRNA purified from rat corpus striatum. Poly(A)$^+$ RNA from the rat striatum was used to first synthesize a cDNA library by reverse transcription using methods well known in the art. The following example shows one method for amplifying the serotonin receptor genes using PCR amplification.

**Example 1: PCR Amplification of Rat Striatum mRNA**

We used a pair of highly degenerate primers, with sequences which were derived from the third and sixth transmembrane (TM) regions of previously cloned G protein-linked receptors in multiple rounds of PCR to amplify the serotonin gene sequences in the striatum library.

Polymerase chain reaction amplification was carried out as previously described (Monsma, F.J., Jr., Mahan, L.C.,

McVittie, L.D., Gerfen, C.R. & Sibley, D.R., Proc. Natl. Acad. Sci. USA 87, 6723-7 (1990)) using cDNA prepared from rat striatal mRNA and 1 μM each of the following primers:

5 5'-GTCGACCCT(GT)T(GT)(CG)GCC(AC)TCA(GT)CA(TC)(GA)G(AG)-TCGCTA-3'

SEQ ID NO: 1

5'-AAGCTTATGAA(AG)AAGGGCAG(GC)CA(AG)CAGAGG(GT)(CT)-(AG)(CA)A-3'.

SEQ ID NO: 2

The PCR timing cycle was 1.5 min at 93°C, 2 min at 50°C,
10 and 4 min at 72°C followed by a 7 min extension at 72°C. The reaction products were purified and size fractionated on a 1% agarose gel. Individual bands were excised, electroeluted, concentrated by ultrafiltration, and ligated into the pCR1000© vector (INVITROGEN). Competent INVaF bacterial cells
15 (INVITROGEN) were transformed and mini-preparations of plasmid DNA prepared for insert sequencing.

This process resulted in the amplification of several cDNA fragments ranging from 300 to approximately 500 base pairs (bp) in size. Each fragment was preliminarily
20 characterized by DNA sequence analysis. Several of these cDNA fragments exhibited sequence homology with previously cloned G-protein coupled receptors such as serotonin, one of which, clone St-B17$_1$ (538 bp), was particularly homologous with several catecholamine receptors and was thus selected for
25 further characterization.

We selected 1 X 10$^6$ recombinants from the rat striatal cDNA library, constructed in the λ ZAP II© vector (STRATAGENE, La Jolla, CA), to be screened with the 538 bp St-B17$_1$ PCR fragment. The 538 bp fragment was made into a probe by $^{32}$P-
30 labeling (via nick translation) using well known methods. Duplicate nitrocellulose filters were hybridized in 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate (5X SSC), 5X Denhardt's solution 0.02 M Na$_2$HPO$_4$, 0.25% SDS, 0.15 mg/ml salmon sperm DNA, and 4 x 10$^6$ dpm/ml of $^{32}$P-labeled probe for
35 24 hr at 37°C. High stringency washing of the filters was performed with 1X SSC and 0.1% SDS at 65°C prior to autoradiography.

The λ phage found to hybridize with the probe were subsequently plaque purified. *In vivo* excision and rescue of the nested pBluescript plasmids from the λ ZAP II clones was performed using helper phage according to the STRATAGENE protocol.

Briefly, the λ phage carry a nested pBluescript plasmid which can be excised via the STRATAGENE protocol. This nested plasmid carried the St-B17 gene and could be used for subsequent sequencing and transfection. Nucleotide sequence analysis was performed using the Sanger dideoxy nucleotide chain termination method with Sequenase© (US BIOCHEMICAL CORP.) on the excised denatured double-stranded plasmid templates.

One positive clone (St-B17$_2$) from the above screening, containing a cDNA insert of 2.8kb, was isolated and nucleotide sequenced. The largest open reading frame was 879 bp long encoding a protein containing sequences homologous to transmembrane (TM) regions I-VI of previously cloned biogenic amine receptors.

Further analysis of the 2.8kb cDNA revealed the presence of a sequence 210 bp beyond the end of the open reading frame which was homologous to the seventh transmembrane (TM) region of several receptors. Careful examination of the DNA sequence between these putative TM regions VI and VII revealed the presence of consensus donor and acceptor sequences for intron/exon splice junctions suggesting the possibility that this region represented an un-spliced intron having an inframe stop codon. Sequence ID NO: 9 is a partial polynucleotide sequence of the 2.8kb clone having the entire coding sequence, and the internal intron. The amino acid sequence corresponding to the first coding region of SEQUENCE ID NO:9 is shown as SEQUENCE ID NO:10. The amino acid sequence corresponding to the second coding region of SEQUENCE ID NO:9 is shown as SEQUENCE ID NO:11.

This would explain the homology to the seventh TM region in the purported non-coding portion of the gene. The possibility that we had isolated a cDNA from a partially spliced mRNA was examined, as described in Example 2 below, using PCR to amplify this putative intron region from cDNA prepared from rat striatal mRNA.

**Example 2: Amplification and Analysis of the 2.8kb Insert**

Primers P3 and P4 were synthesized to have homology near to the 3' and 5' ends of the putative splice region. The sequence of primer P4 is complementary to the coding sequence since it was designed to anneal with the homologous DNA strand. With these primers, we would either isolate 223 bp sequences corresponding to a spliced message, or 416 bp sequences corresponding to an unspliced message. This system could thereby indicate if the putative intron acceptor/donor sites were really utilized for splicing reactions in the cell.

| PRIMER | SEQUENCE | SEQ ID NUMBER |
|--------|----------|---------------|
| P3 | AAGCATAGCA GGAAGGCCTT GAAGGCCAGC CTG | SEQ ID NO: 3 |
| P4 | GGCGAGAAAT ACGCCCTGAA GTTCTCCCGG GAC | SEQ ID NO: 4 |

Amplification across the putative splice region with primers P3 and P4 resulted in a primary DNA product of 223 bp, the size predicted for mRNA spliced at the observed donor/acceptor sites, and a minor band of 416 bp, the size expected based on the sequence of the unspliced St-B17$_2$ cDNA clone. We thus believed that the St-B17$_2$ cDNA clone was derived from a minor, incompletely spliced mRNA.

Since an additional screen of the cDNA library did not result in the isolation of a full-length, completely spliced clone, we used PCR following the protocol outlined in Example 1 to amplify the predicted coding sequence from rat striatal mRNA.

**Example 3: Amplification of Putative Coding Region**

We used primers P1 and P2 (shown below as SEQ ID NOS: 5 and 6, respectively) and PCR to amplify the 2.8kb clone isolated in Example 2 from the rat striatum cDNA library. Primer P2 was complementary to the coding sequence since it was designed to hybridize with the homologous nucleotide strand.

| PRIMER | SEQUENCE | SEQ ID NUMBER |
|--------|----------|---------------|
| P1 | TTGCCAATAC TACTCTAAGG TGCAGCTTCC | SEQ ID NO: 5 |
| P2 | CACACGACTT AACTCCATAG AGTCGATCGG | SEQ ID NO: 6 |

Two nucleotide sequences of 1.6 and 1.4 kb were obtained from the PCR reaction, the latter of which (St-B17₃)was subcloned into plasmid pBluescript II© SK (+). Sequence analysis confirmed the identity of St-B17₃ and revealed that the putative intron was absent in this clone (SEQ ID NO: 7) with the flanking exons spliced together at nucleotide 873 as predicted. Splicing at this position resulted in a 1,311 bp open reading frame encoding a protein of 437 amino acid residues (SEQ ID NO: 8) with a calculated molecular weight of 46.8 kD.

Hydropathy analysis of the deduced amino-acid sequence of the spliced St-B17₃ protein indicated seven hydrophobic regions predicted to represent putative transmembrane spanning domains. When compared to previously cloned G-protein coupled receptors, the transmembrane regions of St-B17 exhibited high homology to various serotonin (5-HT) receptors suggesting that it may be a member of this receptor family.

Within the transmembrane regions, St-B17₃ exhibited homologies of 41%, 40%, 39%, 38%, 37% and 36% with 5-HT₂, 5-HT₁D, 5-HT₁C, 5-HT₁B, 5-HT₁A and 5-HT₁E receptors, respectively. The third cytoplasmic loop of St-B17₃, consisting of 57 residues, would be the shortest of the cloned 5-HT receptors, though similar in length to the 5-HT₁C and 5-HT₂ receptors. These receptors have been described previously by Julius, D. et al., _Science_ 241, 558-564 (1988); and Julius, D. et al., _Proc. Natl. Acad. Sci. USA_ 87, 928-932 (1990); and Pritchett, D.B., et al., _EMBO J._ 7, 4135-4140 (1988). Similarly, while the 117 residue carboxy-terminal tail of St-B17₃ is the longest amongst the 5-HT receptors, it is similar in length to that found in the 5-HT₁C and 5-HT₂ receptors.

The combination of a relatively short third cytoplasmic loop and a long carboxy-terminus is common among receptors which are coupled to the stimulation of either the adenylyl

cyclase or phospholipase C signal transduction systems. St-B17$_3$ also contained one potential N-linked glycosylation site at Asn-9 in the extracellular amino terminus. Additionally, several potential sites for phosphorylation by the cyclic AMP-dependent protein kinase or protein kinase C in both the third cytoplasmic loop and the intracellular carboxy-terminal tail were deduced.

To definitively establish the identity of the receptor encoded by St-B17$_3$, we wanted to express this gene in mammalian cells. Unfortunately, the PCR-amplified full-length, correctly spliced St-B17$_3$ cDNA could not be used directly for expression due to several PCR-generated base substitutions in the 5' region up-stream of the internal Bam H1 site.

We thus constructed a hybrid cDNA in which the entire 3' sequence down-stream of the Bam H1 site from the intron-containing clone (St-B17$_2$) was replaced with the corresponding Bam H1 fragment from the correctly spliced, PCR-amplified cDNA (St-B17$_3$) as explained below in Example 4.

**Example 4: Construction of Expression Clone**

We cleaved the 1.4kb St-B17$_3$ clone isolated in Example 3 with BamHI by well known methods. We also cleaved the 2.8kb St-B17$_2$ clone isolated in Example 1 with BamHI. By mixing and annealing the fragments from these two digestions, we derived a clone (St-B17) with the 5' end of St-B17$_2$ and the 3' end of St-B17$_3$. The resultant fusion gene had a functional 5' leader and initiation codon, and a correctly spliced intron/exon junction.

This construct was subcloned into the eucaryotic expression vector pCD-SRα yielding pSRα-B17. This clone was then used to transfect mammalian cells to study the expression, binding, and regulation of the new serotonin receptor gene as discussed below in Example 5.

**Example 5: Transfection of Mammalian Cells with St-B17**

Transient transfection of Cos-7 cells with pSRα-B17 resulted in the appearance of high affinity and saturable

binding sites for the serotonergic ligand [$^{125}$I]-LSD (lysergic acid diethylamide).

Cos-7 cells were transfected with the pSRα-B17 construct using the calcium phosphate precipitation method as previously described by Monsma, F.J. et al., <u>Proc. Natl. Acad. Sci. USA</u> 87, 6723-7 (1990). Cells were harvested 72 hours after transfection and either disrupted in a dounce homogenizer in 50 mM Tris-HCl, pH 7.4 at 37°C, 10 mM MgSO$_4$ and 0.5 m M EDTA, or frozen in 5 mM Tris-HCl, pH 7.4 at 25°C, 5 mM MgCl$_2$, 250 mM sucrose and stored in liquid N$_2$ prior to membrane preparation. Crude membranes were prepared from cell homogenates by centrifugation at 43,000 x g, and re-suspension in homogenization buffer at a protein concentration of 60 μg/ml.

Once the crude membranes had been prepared from the cell homogenates, we proceeded to pharmacologically screen various drugs for their effect on [$^{125}$I]-LSD to St-B17 as discussed below in Example 6.

**Example 6: Pharmacological Screening Assays Using St-B17**

It was first necessary to determine the binding strength and saturation point of LSD, so that we could later analyze drugs that displaced this chemical. Radiolabeled LSD ([$^{125}$I]-LSD) exhibited a dissociation constant ($K_D$) of 1.26 (±0.17, n=3) nM and maximum binding ($B_{max}$) values ranging from 2-5 pMoles/mg protein (Figure 1) when added to the membranes prepared by the method of Example 5. Figure 1 shows the saturation analysis of [$^{125}$I]-LSD binding to membranes prepared from Cos-7 cells transiently transfected with construct pSRα-B17. The experiment reported is representative of 3 independent experiments conducted in triplicate. The inset (Figure 1) is a scatchard analysis of the saturation binding data. In this experiment the $K_D$ and $B_{max}$ values were 1.5 nM and 3.4 pMoles mg protein$^{-1}$, respectively. This assay shows the strong binding of LSD to the St-B17 receptor.

Figure 2 displays the results of a pharmacological competition assay to analyze the displacement of [$^{125}$I]-LSD in the presence of various serotonin receptor binding drugs. This experiment was performed on Cos-7 cell membranes

expressing the St-B17 serotonin receptor. The competition curves shown are representative of 3 independent experiments conducted in triplicate. Average inhibition constants ($K_i$) and SEM values are given in Table 1. No specific binding of

5        $[^{125}I]$-LSD was observed in untransfected Cos-7 cells or in cells transfected with the pCD-SRα expression vector alone (controls).

TABLE 1.   Pharmacology of St-B17 Expressed in Cos-7 Cells

| Drug | $K_i$ (nM) Mean ± SEM | |
|---|---|---|
| | vs. $[^{125}I]$-LSD | vs. $[^3H]$-5-HT |
| Methiothepin | 1.84 ± 0.23 | 0.39 ± 0.063 |
| Lisuride | 8.19 ± 0.48 | 5.3 ± 1.3 |
| Clozapine | 12.87 ± 1.69 | 20 ± 2.5 |
| Dihydroergotamine | 13.07 ± 0.90 | 5.4 ± 5.4 |
| 2-Br-LSD | 17.14 ± 1.11 | |
| Pergolide | 29.87 ± 7.31 | |
| Metergoline | 29.97 ± 0.38 | 61 ± 23 |
| Amoxipine | 30.40 ± 1.64 | |
| Lergotrile | 36.45 ± 1.04 | |
| 5-methoxytryptamine | 38.92 ± 3.95 | 18 ± 2 |
| Ritanerin | 44.12 ± 4.35 | 16 ± 2 |
| Mianserin | 45.74 ± 8.93 | 38 ± 7 |
| Clomiprimine | 53.78 ± 3.28 | |
| 5-Hydroxy-N,ω-methyltryptamine | 57.95 ± 8.32 | |
| Loxapine | 64.75 ± 0.60 | 56 ± 1.5 |
| Amitriptyline | 69.64 ± 7.21 | 82 ± 6 |
| N,N-Dimethyl-5-methoxytryptamine | 79.81 ± 3.86 | |
| 1(1-naphthyl)piperazine | 103.61 ± 13.81 | |
| 5-Benzyloxytryptamine | 110.18 ± 17.90 | |
| Cyproheptadine | 133.85 ± 6.17 | |
| Doxepin | 135.74 ± 7.11 | |
| Nortriptyline | 147.49 ± 2.61 | |
| 5-HT | 151.01 ± 12.78 | 56 ± 9 |
| Dihydroergocryptine | 160.70 ± 13.21 | |
| Imipramine | 208.49 ± 22.76 | 190 ± 3 |
| N,ω-Methyltryptamine | 341.60 ± 32.21 | |
| MethyscrGide | 371.74 ± 72.66 | |
| Tryptamine | 438.27 ± 14.84 | |
| TFMPP | 482.37 ± 37.21 | |
| CGS 12066B | 737.69 ± 66.63 | |
| 5-Carboxamidotryptamine | 774.35 ± 84.39 | 253 ± 20 |
| PAPP | 805.62 ± 75.17 | |
| Mesulergine | 1718.88 ± 248.35 | |
| Fluoxetine | 1765.36 ± 290.37 | |
| mCPP | 2309.60 ± 343.05 | |
| 2-MPP | 3054.95 ± 321.06 | |

Increasing concentrations of the indicated compounds were used to inhibit the binding of either 0.5 nM [$^{125}$I]-LSD or 5 nM [$^3$H]-5-HT to membranes of Cos-7 cells transiently transfected with clone St-B17. [$^{125}$I]-LSD competition assays were performed by incubating the transfected Cos-7 cells with [$^{125}$I]-LSD and the indicated concentrations of each drug as described above and in Figure 2, while those for [$^3$H]-5-HT were performed as previously described (Hamblin, M.W. & Metcalf, M.A., Mol. Pharmacol. 40, 143-148 (1991)). $K_i$ values were obtained from graphically determined $IC_{50}$ values by the method of Cheng and Prussoff (Biochem. Pharmacol. 22, 3099-3108 (1973)) and are presented as the geometric mean ± SEM (n = 3) calculated according to De Lean et al. (Mol. Pharmacol. 21, 5-16 (1982)).

This method thereby provides a way of screening drug candidates for central nervous system activity by testing its binding to isolated St-B17. We believe that drugs which bind to the St-B17 receptor *in vitro* will also have effects *in vivo*. In addition to directly measuring the binding of a drug to the St-B17 receptor, we were also able to measure the displacement of known binding ligands to the St-B17 receptor. By measuring the binding of a ligand as discussed above, and then comparing that binding to the level of binding in the presence of a drug candidate, we were able to estimate the potential of the drug to displace the ligand.

The following compounds were screened and found to exhibit inhibition constant ($K_i$) values >10$\mu$M: zimelidine, zacopride, NAN-190, citalopram, BIMU 1, metoclopromide, fenfluramine, MDL 72222, 8-OH-DPAT, BRL 24924, BRL 43694A, GR 38032F, ICS 205,930, ketanserin, melatonin, spiroperidol, tyramine, DAU 6215, DAU 6285, dopamine, epinephrine, histamine, idazoxan, LY 278,584, maxindol, norepinephrine, octopamine, paroxetine, and pindolol.

Preliminary characterization of the St-B17 pharmacology indicated that amongst several endogenous biogenic amines, including dopamine, melatonin, epinephrine, norepinephrine or histamine, only 5-HT (serotonin) was capable of completely

displacing $[^{125}I]$-LSD binding, exhibiting a $K_i$ of 150 nM (Figure 2 and Table 1). The Hill coefficient for the 5-HT competition curve was not significantly different from unity and the affinity of 5-HT was not influenced by addition of the guanine nucleotide analogue GppNHp.

The binding of 5-HT to St-B17 was also investigated directly using $[^{3}H]$-5-HT as the radioligand. Saturation analysis of $[^{3}H]$-5-HT binding revealed the presence of a single class of high affinity, saturable binding sites in transfected, but not untransfected, Cos-7 cells. Analysis of the $[^{3}H]$-5-HT saturation binding data revealed a $K_D$ of 37 ($\pm 5.0$, n=3) nM and $B_{max}$ values of 1-3 pMoles/mg protein. The binding of $[^{3}H]$-5-HT was similarly not affected by the addition of guanine nucleotides. These initial binding data would thus suggest that St-B17 encodes a 5-HT receptor subtype.

Further characterization of the St-B17 pharmacology involved the utilization of a variety of drugs which are known to exhibit specificity for various serotonergic receptor subtypes and other binding sites. The average $K_i$ values for compounds competing with better than 10 $\mu$M affinity are shown in Table 1 with representative competition curves for $[^{125}I]$-LSD binding shown in Figure 2.

Examination of the rank order of potency for a variety of serotonergic agents reveals that the pharmacology of clone St-B17 does not correspond to any previously described serotonin receptor subtype. A number of drugs selective for 5-HT$_3$ and 5-HT$_4$ receptors (i.e., MDL 72222, ICS 205,930, or DAU 6285) exhibit virtually no affinity for St-B17, while agents selective for other 5-HT receptor subtypes such as 8-OH-DPAT (5HT$_{1A}$), CGS 12066B (5-HT$_{1B}$), mesulergine (5-HT$_{1C}$), or ketanserin (5-HT$_2$) bind with relatively low affinity. Ergot alkaloids, especially ergoline derivatives (i.e., LSD, lisuride, or pergolide), display relatively high affinity for St-B17 as does the non-selective serotonergic antagonist methiothepin.

Interestingly, the atypical and typical anti-psychotics clozapine and loxapine, respectively, also exhibited high affinity for St-B17, as did several tricyclic anti-depressant drugs (i.e., amoxipine, clomiprimine, and amitriptyline) which all had $K_i$ values under 100 nM. In general, the drugs which exhibited the greatest affinity for St-B17 (i.e., $K_i$<100 nM) were tricyclic, ergoline or tryptamine derivatives.

Competition for [$^3$H]-5-HT binding by a number of drugs revealed, with a few exceptions, the same rank order of potency as for inhibition of [$^{125}$I]-LSD binding (Table 1). However, for some drugs the $K_i$ values determined by competition with [$^3$H]-5-HT were up to 5-fold lower than those determined by competition with [$^{125}$I]-LSD, with the exception of clozapine, metergoline and amitriptyline, which exhibited somewhat greater potency in competition with [$^{125}$I]-LSD.

Although the pharmacological profile of St-B17 did not correspond to previously defined 5-HT receptor subtypes, it did resemble the profile described by Conner and Mansour for 5-HT stimulation of adenylyl cyclase activity in the NCB-20 neuroblastoma cell line (Mol. Pharmacol. (1990) 37, 742-751). In these cells 5-HT activated adenylyl cyclase with an $EC_{50}$ of 300 nM and a variety of tryptamine derivatives exhibit $EC_{50}$ values similar to their $K_i$ values for inhibition of [$^{125}$I]-LSD binding to St-B17. Similarly, antagonism of 5-HT-stimulated adenylyl cyclase activity in NCB-20 cells by a variety of compounds, including methiothepin, mianserin, amitriptyline, and cyproheptadine, also exhibited apparent $K_i$ values and a rank order of potency similar to those observed for inhibition of [$^{125}$I]-LSD binding to St-B17.

It is contemplated that other means of labeling LSD or the other tested drugs would also provide an assay for the inhibition of compounds such as that discussed above. LSD could be labeled either radioactively or colormetrically and still provide the desired assay.

The distribution of mRNA encoding St-B17 was evaluated by Northern blot analysis of poly (A+) RNA prepared from a variety of rat brain regions as well as other peripheral

tissues. · The results from these experiments are shown below in Example 7.

Example 7:  Distribution of mRNA Encoding St-B17

Figure 3 shows a Northern blot analysis of St-B17 RNA transcripts in rat brain.  Each lane contained 3 μg of poly (A+) RNA.   Lanes: AMYG, amygdala; CB, cerebellum; CTX, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; MED, medulla; OB, olfactory bulb; OT, olfactory tubercle; PIT, pituitary; RET, retina; STR, striatum; THAL, thalamus. Locations of RNA size (kb) markers are indicated.

To prepare the Northern blot, poly (A+) RNA was prepared from rat brain regions using the FASTTRACK© mRNA isolation system (INVITROGEN).  3 μg of poly (A+) RNA was denatured and subjected to electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde.  RNA was transferred to a nylon membrane (Genescreen Plus, ·Dupont) by capillary transfer and immobilized by UV crosslinking.  Northern blots were probed with the entire 2.8 kb cDNA insert of clone St-B17 [$^{32}$P]-labeled by the random primer method.  Northern blots were hybridized with 3 x 10$^6$ dpm/ml of probe in 1 M NaCl, 1% SDS, 10% dextran sulfate at 60°C for 18 hr.  Blots were washed in 2X SSC/1% SDS at room temperature, followed by 0.2X SSC/1% SDS at 65°C, and were exposed with an intensifying screen for 15 days at -70°C.

A single transcript of 4.2 kb was observed in various brain regions with highest expression appearing to occur in the corpus striatum (Figure 3).  St-B17 mRNA was also observed in the amygdala, cerebral cortex and olfactory tubercle whereas it was absent, or present in undetectable levels, in the cerebellum, hippocampus, hypothalamus, medulla, olfactory bulb, pituitary, retina and thalamus.   Similarly, no transcript was observed, even after prolonged exposures, in Northern blots of mRNA from rat heart, lung, kidney, spleen, pancreas, smooth muscle, skeletal muscle, stomach, ovary, prostrate, or testes (data not shown).

This result indicates that the St-B17 receptor is produced in much higher levels in the corpus striatum than in

other regions of the brain. In addition, the receptor is undetectable in non-brain tissues.

The localization of St-B17 mRNA to limbic and cortical regions, and the relatively potent interaction of several therapeutically important drugs, including the atypical anti-psychotic clozapine, the typical anti-psychotic loxapine, and the tricyclic anti-depressants amoxipine, clomiprimine, amitriptyline, and nortriptyline with St-B17 suggests that this receptor may play an important though hitherto unappreciated role in several neuropsychiatric disorders which involve serotonergic systems.

In addition to the mRNA distribution of St-B17, we were also interested in the intracellular cascade of events following agonist binding to the receptor. One possible mechanism of receptor signaling involved adenylate cyclase activity. To investigate this possibility, the following experiment was performed.

**Example 8: Analysis of Receptor Mediated cAMP Activity**

Human Embryonic Kidney cells (HEK 293 available from the ATCC) were stably co-transfected in 150mm dishes by the $CaPO_4$ technique with 30 $\mu$g of pSR$\alpha$-B17 and 3$\mu$g of pMam-neo (INVITROGEN). These HEK 293 cells were then subjected to selection with 600 $\mu$g/ml genetecin (G418) (GIBCO). The resistant colonies were collected and screened for receptor expression by [$^{125}$I]-LSD binding as described in Example 6. One clone was isolated which expressed approximately 800 fMoles/mg St-B17 receptor protein and was subsequently used for assessment of cAMP accumulation.

Intracellular cAMP levels were determined after a 5 minute incubation of intact cells with various test compounds at 37°C in the presence of the cAMP phosphodiesterase inhibitor RO-20-1724, using a modification of a previously described cAMP assay (Monsma, F.J., Jr., Barton, A.C., and Sibley, D.R. (1990) _J. Neurochemistry_ 54, 1200-1207) wherein the adrenal cAMP binding protein was replaced with 0.8mg/ml protein kinase A in water.

As shown in Figure 4, serotonin causes a potent dose-dependent increase in cAMP levels in transfected HEK-293 cells with an average 50% effective concentration ($EC_{50}$) of 145 ($\pm40$, n=3) nM, whereas there was no detectable response in un-transfected cells (data not shown). This suggests that the St-B17 receptor mediates some part of its cell signaling through a cAMP-related pathway.

A pharmacological analysis of the cAMP response (Figure 5) indicates that the serotonergic agonists 5-MT and 5-CT are also able to elicit an increase in cAMP levels. The ergot alkaloids lisuride and dihydroergotamine also stimulate cAMP accumulation, although these drugs appear to function as partial agonists to the St-B17 receptor. Amoxipine, methiothepin and clozapine (Figure 5) all appear to act as antagonists of this receptor as they had no significant effect on cAMP levels on their own, but were able to substantially inhibit the response elicited by 5-HT. These data suggest that the St-B17 receptor is functionally linked to the adenylyl cyclase signal transduction system.

The measurement of cAMP levels in a cell transfected with St-B17 can thereby provide an assay for detecting potential agonists and antagonists of serotonin receptors. Using the above method, we can analyze the accumulated level of cAMP in the cell after treatment with a drug to be tested. A lowering of the cAMP level in the presence of 5-HT indicates a drug which has inhibitory affects on 5-HT receptor binding.

We were also interested in isolating the human variation of the present invention. Our method of isolating the human receptor is discussed below.

**Example 9: Isolation of the Human St-B17 Receptor**

Two different restriction fragments derived from the rat St-B17 gene were used as probes to retrieve the human homolog. A 1192 base-pair Xma I-BstXI fragment and a 655 base-pair Bam HI-EagI fragment of rat St-B17 were labeled with [$\alpha^{32}$P]-dCTP using the well known nick-translation method. These labeled fragments were then used to screen a commercially available human genomic library (Stratagene #946205).

The library was plated onto host cells C600 and PLK17 respectively, and duplicate nylon (Nytran, Schleicher and Schuell) lifts made. Each of the labeled fragments was hybridized (6 X SSC at 55°C, pH7) and washed (2 X SSC, 58°C) at moderate stringency. Positive isolates were plaque purified through a second round of hybridization.

An insert from each positive plaque isolate was excised by EcoRI digestion, and then subcloned into a corresponding site in the phagemid vector pBluescript SK(-) (Stratagene). These inserts were then digested with several restriction enzymes, and run on a Southern Blot. Fragments hybridizing to labeled St-B17 rat sequences were identified by Southern hybridization. Hybridizing fragments were then subcloned into pBluescript SK(-) as discussed above. The nucleic acid sequence of these inserts was then determined by dideoxynucleotide termination using Sequenase (United States Biochemicals) in conjunction with standard subcloning techniques. The nucleotide sequence of the human St-B17 gene is SEQUENCE ID NO:12. The corresponding amino acid sequence is SEQUENCE ID NO: 13.

It should be understood that all of the previous experiments relating to identification of binding ligands to the rat receptor can be performed in a similar fashion with the human gene. For instance, the human gene can be cloned into an expression plasmid and placed in COS-7 cells by methods similar to those discussed above, and used to assay binding of competitors such as those revealed in Table 1. This would provide a method of assaying inhibitors of serotonin binding to the St-B17 receptor.

Specifically, Example 8 can be repeated with the human receptor to reveal if a particular compound was mimicking or inhibiting the binding of serotonin by measurement of the relative level of cAMP before and after treatment with the compound. The methods of this example can also be repeated with cDNA of other mammals or other vertebrates to obtain the corresponding St-B17 receptor from these organisms.

Other experiments are also contemplated relating to discovering which cells in vivo express the receptor gene on

their surface. To allow us the ability to study the expression of the receptor on cell surfaces throughout the body, we needed to produce antibodies against the receptor protein. A method for producing antibodies is discussed below

5   in Example 10.

**Example 10: Production of Antibodies Against St-B17**

COS-7 cells expressing the St-B17 receptor protein derived in Examples 11 and 12 are lysed with NP40, and the isolated membranes are injected into rabbits. The lysed

10   membranes are isolated in a non-ionic detergent so as not to affect the membrane bound receptors. Freunds adjuvant is used in the injection to help stimulate an antigenic response by the rabbits. After two booster shots of the lysed membranes, the rabbits are bled and the sera isolated by centrifugation.

15   The antibodies in the crude rabbit sera extract are $^{125}$I labeled by well known methods, and tested for activity against the transfected COS-7 cells. A western blot having one lane containing proteins from transfected cell lysates, and a second lane having untransfected lysates (control) is run. A

20   strong band indicating antibody binding at 46.8kd in the transfected cell lane, that is not apparent in the untransfected lane shows that polyclonal antibodies against the St-B17 receptor protein have been properly isolated.

Monoclonal antibodies can be made by well known methods

25   in addition to the polyclonal antibodies discussed above. One method of producing monoclonal antibodies is discussed below in Example 11.

**Example 11: Production of Monoclonal Antibodies**
**Against St-B17**

30   The St-B17 transfected COS-7 cells produced in the previous examples are lysed with NP-40 and the cell membranes are pelleted by centrifugation. The isolated membranes, having bound St-B17 receptor proteins are injected in Freunds adjuvant into mice. After being injected 9 times over a three

35   week period, the mice spleens are removed and resuspended in PBS.

The suspended spleen cells are mixed (approximately 4:1) with SP 2/0 Myeloma cells. Polyethylene glycol is added to fuse the myeloma cells to the spleen cells, and the fused cells are selected in HAT media. The fused cells are aliquoted so that only one cell is grown in each well of a 96 well microtiter plate. Each cell is grown and the media removed and secreted proteins are $^{125}$I labeled. The labeled media from each well is used to probe a Western blot of transfected and untransfected COS-7 cells (see Example 12). The desired fusion cell will produce a monoclonal antibody that strongly binds a 46.8kD band in the transfected COS-7 cell lane on the Western blot, but doesn't bind to any other protein in that lane, or the control lane.

This method provides a way of detecting expression of the St-B17 serotonin receptor protein. Another method of detecting expression of St-B17 is by in situ hybridization as discussed below in Example 12.

Example 12:   In Situ Hybridization of St-B17

In situ hybridization allows the identification of mRNA within intact tissues, such as the rat brain. In this method, oligonucleotides corresponding to unique portions of the St-B17 gene (SEQ ID NO: 7) are used to detect specific mRNA species in the brain.

An anesthetized rat is transcardially perfused with cold PBS (5-20 minutes), followed by perfusion with a 4% formaldehyde solution. The brain is removed, frozen in liquid nitrogen, and cut into 5 $\mu$m to 30 $\mu$m sections. The sections are placed on slides and incubated in proteinase K for approximately 15 minutes. The slides are then rinsed in DEP, water, and ethanol, and placed in a prehybridization buffer.

A radioactive probe corresponding to primer P1 is made by nick translation and incubated with the sectioned brain tissue. After incubation and air drying, the labeled areas are visualized by autoradiography. Dark spots on the tissue sample indicate hybridization of the probe with the brain mRNA thereby demonstrating expression of the St-B17 receptor.

All of the above cited references are herein incorporated by reference. The present invention should not be limited by these examples but only by the following claims.